

Department of Microbiology, Eötvös L. University,
Budapest, Hungary

On the community-structure of the gut-microbiota of *Eisenia lucens* (Annelida, Oligochaeta)

K. MÁRIALIGETI

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1. Introduction

As it is well known, Gram-negative bacteria are common inhabitants of the intestinal canal of soil animals, including earthworms, and they can influence in many ways the host-animals' activities, and indirectly the soil forming processes (KHAMBATA & BHAT 1957; SZABÓ 1974; REYES & TIEDJE 1976). Unfortunately, little is known about their taxonomic identity at generic or species level, presumably because of the considerable difficulties involved in their correct determination. Members of the genus *Vibrio*, for example, are perhaps frequent in the digestive canal of earthworms but up till now only very few data confirm this supposition, and a *Pheretima* sp. is the only from which the presence of a *Vibrio* sp. actually has been shown (KHAMBATA & BHAT 1957).

True vibrios are organisms of great sanitary importance because of their pathogenic properties for man and for various animals. Vibrios occur otherwise worldwide in salt waters and perhaps in fresh ones, furthermore in the alimentary canal of man and certain animals. The interest in the assay of genus *Vibrio* has been focussed on the pathogenic species, in particular on *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio alginolyticus* (SAKAZAKI 1968; described as *Beneckia alginolytica* by BAUMANN et al. 1971) which have been extensively studied as causative agents of food infection (acute gastroenteritis) following consumption of raw or semipreserved sea or other food (SAKAZAKI 1969; FUJINO et al. 1974). *Vibrio alginolyticus* has been incriminated in infections of wounds, ears and eyes in persons who had been in contact with marine or estuarine shore areas (ROLAND 1970; 1971; etc.).

There are only few known separate types or species in the genus *Vibrio* the occurrence and growth of which were not detected in some way or other in connection with marine or at least true aquatic habitats. These "terrestrial" types are either pathogenic organisms or harmless ones, being mostly associated with internal animal milieus. *Vibrio succinogenes* (WOLIN et al. 1961) is, for example, an unusual anaerobe that was originally isolated from bovine rumen. Recently PALENSTEIN HELDERMAN et al. (1976) isolated microaerophilic, motile, slightly curved Gram-negative rods from the gingival crevice of patients with gingivitis, resembling *Vibrio succinogenes*, etc.

Studies on the distribution and ecological importance of vibrios in soil, plant and animal habitats were limited by many difficulties and unsolved problems of *Vibrio* taxonomy. Although today we generally consider all bacteria characterized by curved Gram-negative, Kovács oxidase-positive cells which are motile with a single polar flagellum, have both respiratory and fermentative (acid without gas produced from carbohydrates in HUGH and LEIFSON medium) metabolism, and are sensitive against pteridine (0—129) as the members of the genus *Vibrio*, yet serious problems can be encountered not only in differentiation and specification within the genus *Vibrio* but also in separating this genus as a whole from other related genera such as are belonging to families Pseudomonadaceae and Enterobacteriaceae (COLWELL 1970).

The purpose of this study is to demonstrate the common occurrence of a *Vibrio* sp. in the intestinal canal of *Eisenia lucens*, a facultative wood digesting earthworm species, and at the same time to contribute to our knowledge on the distribution and biology of vibrios in internal animal habitats of the soil ecosystem.

2. Materials and methods

2.1. Study area

The sampling site was in the "Bükk"-mountains, near Lillafüred (in Hungary) in a mixed beech forest (*Quercus petraeae*-*Carpinetum*) stand settled on brown-forest soil developed from limestone as parent rock. The specimens of *Eisenia lucens* were collected from the easily fragmentable destroying stocks of beech (*Fagus sylvatica*), in which they are tunneling in large numbers.

2.2. Identification of the collected specimens

Systematic identification of specimens used for bacteriological investigations was carried out on the basis of their characteristic external features (VEJDovsky 1875). We are very grateful to Prof. A. Zicsi (Dept. of Syst. Zoology, Eötvös L. University, Budapest, Hungary) for checking the validity of our species-identification and studying the *Eisenia* material collected by us.

2.3. Sampling of intestinal contents

Only the hind-gut content was investigated, which harbours a more characteristic microflora than that of the mid-gut. The collection of worms and the sampling of the hind-gut contents were carried out aseptically at their natural habitats. The surface disinfected worms (Neomagnol was used) were gently massaged with sterile forceps to obtain fresh fecal material. Excrement samples were transported to the laboratory for examination within 5 hr after collection.

2.4. Plating of samples and isolation of bacteria

Weighed samples of intestinal contents were suspended and serially diluted (10^{-1} to 10^{-6}) in sterile tap water, and 0.1 ml amounts of each dilution were plated on nutrient agar and synthetic-glucose agar, respectively, using the surface spread technique. Nutrient agar: meat extract (Lab-Lemco Powder) 3.0 g; peptone (Difco) 5.0 g; purified agar (Difco) 20.0 g; distilled water 1000 ml. Synthetic-glucose agar: glucose 10.0 g; $(\text{NH}_4)_2\text{HPO}_4$ 4.0 g; NaCl 5.0 g; K_2HPO_4 2.0 g; MgSO_4 1.0 g; CaCl_2 0.4 g; $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ 0.02 g; $\text{MnSO}_4 \times 7\text{H}_2\text{O}$ 0.01 g; purified agar (Difco) 20.0 g; distilled water 1000 ml. The pH adjusted to 7.0 and the media were sterilized by autoclaving for 15 minutes at 1.0 atm¹). Five to twenty-five plates for each dilutions and media were prepared in Petri-dishes. Inoculated plates were incubated at 28 °C for 48–72 hrs. Colonies were counted after incubation. The isolations were made in large number and in a random manner, transferring colonies from the above media to nutrient slants.

2.5. Grouping method and purity checking

The isolated strains were compared on the basis of a number of easily accessible cultural and morphological characters and biochemical tests. The similar strains were collected into groups, and from each group representative strains were selected for subsequent detailed taxonomic investigations.

The strains were tested for purity by investigating their macroscopical and microscopical morphology, and certain selected physiological features. Strains which proved to be nonhomogeneous were reisolated.

2.6. Diagnostic features studied

Stock cultures were maintained on nutrient agar slopes kept in the refrigerator at 4–6 °C. Subcultures were made at intervals of 2–4 weeks. The inoculated slants were incubated at 28 °C for 24 hr. As a standard inoculum served a loopful of a culture grown on nutrient slants for 24 hr, or a drop of one loopful of bacteria suspended in 10 ml sterile distilled water, and sometimes a drop from a 24 hr old nutrient broth culture. Tests were carried out in duplicate at incubation temperatures of 28 °C except where otherwise reported:

(1) Colony morphology. A loopful of a nutrient broth culture was inoculated onto synthetic agar plate and incubated for 24 to 72 hrs. The colonies were examined with a binocular stereo microscope; capability for growth on this medium was also reported.

(2) Cell morphology. Micromorphological characteristics scored from 24 hr cultures grown in nutrient broth, and examined by phase-contrast microscopy.

1) 1.0 atm = $1.01325 \cdot 10^5$ Pa

(3) Motility was scored from wet-mount preparations examined by phase-contrast microscopy. Flagella stains were prepared by the method of LEIFSON (1951).

(4) Gram stains were performed by the modification of HUCKER (1923).

(5) Sensitivity to antibiotics and antibacterial substances, and the vibriostatic compound (2—4 diamino, 6—7 diisopropyl-pteridine) described by SCHUBERT (1962) were tested. Seeded nutrient agar plates were used with sensitivity discs (made by the Serobacteriological Institute Human, Budapest, Hungary) placed on their surface, for the following antibiotics and antibacterial compounds: penicillin (3 IU), oxacillin (10 µg), methicillin (20 µg), chloramphenicol (30 µg), oleandomycin (30 µg), streptomycin (30 µg), tetracycline (30 µg), neomycin (100 µg), polymyxin-B (15 µg), erythromycin (10 µg), superseptyl (400 µg), nitrofurantoin (300 µg), chlortetracycline (30 µg), oxytetracycline (30 µg), vancomycin (50 µg), kanamycin (30 µg), spiramycin (30 µg), novobiocin (30 µg), ampicillin (20 µg), colistin (20 µg), lincomycin (10 µg), cephalosporin (10 µg), pristnamycin (10 µg), nalidixic acid (30 µg), paramomycin (50 µg), gentamycin (20 µg), carbenicillin (50 µg), nystatin (100 IU), and the vibriostatic compound (20 µg). The sensitivity was reported by measuring the radius of the inhibition zone.

(6) Catalase production. Ten per cent hydrogen peroxide was dropped on 24 hr old slant cultures. Gas production was recorded.

(7) Oxidase test. The method of Kovács (1956) was used.

(8) The utilisation of citrates as sole source of carbon was tested on Difco Simmons' citrate agar.

(9) Nitrate reduction was examined in Difco nitrate broth. Nitrite was shown after 5 days incubation, with GRIESS-ILOSVAY reagent. Reduction beyond nitrite was tested with the zinc dust method.

(10) Hydrogen sulfide production from cysteine was demonstrated by using lead acetate paper strips.

(11) Indole formation from tryptophane was shown by the Kovács reagent from two days old broth cultures.

(12) The MR and VP tests were revealed from glucose-phosphate medium. The detection of acethyl-methylcartinol was made by BARRITTS' method (1936).

(13) The capability for growth in nutrient broth at high pH values: the medium was adjusted after autoclaving with sterile diluted NaOH to pH 9.5.

(14) Ammonia production from peptone was traced by treatment of two-day-old peptone-water cultures for Nessler reagent.

(15) Arginine hydrolysis. After incubation for five days in arginine-glucose medium, ammonia was tested by Nessler reagent.

(16) The proteolytic activity was determined in stab inoculated nutrient gelatin. This experiment lasted for 4 weeks.

(17) Temperature range of growth: the growth was observed at temperatures of 37, 45 and 50 °C.

(18) Hydrolysis of starch was tested upon nutrient agar containing 0.2% soluble starch by flooding with lugol solution after 5 days incubation.

(19) The hydrolysis of aesculin was detected by the method of SNEATH (1956).

(20) Cleavage of tweens (tween 40, 60 and 80) was recorded by Sierras' method (1957).

(21) Casein hydrolysis was tested with 10% skimmed milk in nutrient agar and flooding the two-day-old growth with acid mercuric chloride.

(22) Oxidation-fermentation tests of dextrose, lactose, mannitol, arabinose, xylose, sucrose was performed in Hugh-Leifson medium. All carbohydrates were added aseptically as Seitz filtered solutions (HUGH and LEIFSON 1953).

(23) Haemolytic activity was determined on nutrient agar plates complemented with 1% sterile defibrinated cattle blood and Alsever solution treated sheep blood, respectively.

(24) The utilization of different cellulose preparations was tested on agar plates of the following composition: NaCl 3.0 g; KH₂PO₄ 1.0 g; MgSO₄ 0.05 g; CaCl₂ 0.05 g; yeast-extract (Difco) 0.5 g; purified agar (Difco) 20.0 g; cellulose 10.0 g; distilled water 1000 ml. The pH adjusted to 7.0, and the media were autoclaved for 15 minutes at 1.0 atm pressure¹⁾. The following preparations were used: Difco cellulose, MN 300 cellulose powder (Machery-Nagel Co.), carboxy-methyl cellulose (Reanal, Hungary).

(25) The ability of lignine cleavage was traced on lignine agar plates by observing the clear zones round the colonies (Freudenberg lignine preparation was used). The medium was as follows: glucose 5.0 g; (NH₄)₂SO₄ 1.3 g; K₂HPO₄ 1.0 g; KH₂PO₄ 0.6 g; purified agar (Difco) 20.0 g; lignine 1.0 g; distilled water 1000 ml. The pH adjusted to 7.0, and the medium was sterilized by autoclaving at 1.0 atm pressure¹⁾ for 15 minutes.

(26) Utilization of vanillin and syrigaldehyde as sole sources of carbon was tested on the following medium described by TSUKAMURA (1975): (NH₄)₂SO₄ 2.64 g; KH₂PO₄ 0.5 g; MgSO₄ × 7H₂O 0.5 g; purified agar (Difco) 20.0 g; distilled water 1000 ml; the pH adjusted to 7.0 with KOH solution. The SEITZ filtered carbon source was added aseptically to the basal medium at a final concentration of 0.02%.

1) 1.0 atm = 1.01325 · 10⁵ Pa

3. Results and discussion

The total number of bacteria measured in the collective samples of fresh hind-gut contents of *Eisenia lucens* is relatively high (about 6×10^7 /g wet faecal material, enumerated on nutrient agar and about 9.5×10^6 /g wet faecal material, calculated using synthetic agar as plating medium, respectively) which clearly shows the highly intense microbial activities in the worms' intestinal canal. Although, as it was subsequently detected, the predominant types of the but bacterial flora are able to grow on synthetic medium too, yet the cells of these types show large differences concerning the required time for their adaptation to inorganic N-sources, these differences being sufficient to cause considerably lower bacterial counts on synthetic medium after a given time of incubation.

As a result of the isolation (on a random basis) of colonies from the faeces infected plates, a total of 473 intestinal bacterial strains were obtained, purified and subjected to the grouping procedure using easily distinguishable cultural, morphological and certain physiological features. This grouping resulted in the separation of 12 groups of similar organisms, as follows.

Group 1. 7 strains of Actinomycetes, mainly *Streptomyces* spp. Their relative number among the isolated and investigated strains was low. But further investigation is needed to clarify the frequency of occurrence of these organisms in the gut of *E. lucens* because the media used and the short time of incubation employed were inadequate to enumerate them with enough certainty.

Group 2 and 3. 13 strains of *Flavobacterium*-like organisms. They produce typically yellow coloured colonies with entire margin.

Group 4. A homogenous group of 21 very similar strains, that belong to the genus *Bacillus*, showing great resemblance to the members of the *B. cereus* group. They are sporadically occurring presumably allochthonous organisms in the intestines of worms.

Group 5. Two isolates of *B. cereus* var. *mycoides* with insignificant frequency of occurrence.

Group 6. Five further sporadically occurring, very similar *Bacillus* strains.

Group 7. A group of 7 red-pigmented bacterial strains with low frequency of occurrence.

Group 8. Another small group of red-pigmented bacteria containing 16 very similar strains which however are not identical with the members of group 7. In the intestine they seem to be subordinate cohabitants of the microbiota.

Group 9. From among the 111 strains isolated from synthetic agar a total of 87 strains (78 %) were clustered together into this group, that forms an assemblage of very similar organisms and represent a predominant member of the gut-microbiota. Thirty-two strains were sorted as representative ones.

Group 10. From among the 362 strains isolated from nutrient agar plates 257 strain (72 %) proved to be very similar. They were clustered together as a group of such strains which are the representatives of the most common microorganism in the hind-gut. These strains showed also a considerable resemblance to the members of group 9. Fifty-five representative strains were selected.

Group 11. Sixteen strains proved to be the members of a variant of the type forming group 10. Five strains were selected for further investigations.

Group 12. A very heterogeneous group, constituted of 33 strains. This group is an assemblage of sporadically occurring, presumedly allochthonous organisms.

In order to clarify the systematic position of the members of the groups of predominant organisms (groups 9, 10 and 11) and to compare them a total of 90 selected representative strains were thoroughly investigated on the basis of the most important diagnostic features.

According to the results of our comparative investigations (Table 1), all members of the predominant groups (9, 10 and 11) form only one large assemblage of strains of a single species which dominates the intestinal flora and can produce enormous cell masses in the gut milieu. The sensitivity of these strains to antibiotics and antibacterial substances was very similar (Table 2): they were most sensitive against the broad spectrum antibiotics

Table 1. Selected physiological and biochemical properties of 90 strains representing the dominating bacterial fraction of the gut community of *Eisenia lucens*

Characteristics	Strains giving positive reactions	
	No. of strains	Per cent of positive strains
Gram-negative, asporogenous	90	100
Catalase	90	100
Oxidase	83	92.3
Simmons' citrate	89	98.8
Growth on synthetic agar	89	98.8
Nitrate to nitrite	83	92.3
Hydrogen sulfide	87	96.6
Indole	0	0
Methyl red	0	0
Voges-Proskauer	0	0
Growth in nutrient broth		
with 2% NaCl	90	100
4% NaCl	90	100
10% NaCl	57	63.3
Growth at		
37 °C	63	70
45 °C	3	3.4
50 °C	0	0
Growth at pH 9.5	69	76.6
Urease	88	97.7
NH ₃ from peptone	0	0
Gelatinase	15	16.7
Hydrolysis of arginine	90	100
Starch hydrolysis	0	0
Casein hydrolysis	7	7.8
Hydrolysis of aesculin	40	44.4
Tween 40	84	93.3
Tween 60	86	95.5
Tween 80	79	87.7
Acid from		
dextrose	76	84.5
lactose	42	46.7
mannitol	46	51.1
arabinose	90	100
xylose	90	100
sucrose	3	3.4
Acid under petrolatum seal from		
dextrose	65	72.2
lactose	42	46.7
mannitol	51	56.7
arabinose	90	100
xylose	90	100
sucrose	21	23.3
Utilization of		
Difco cellulose	0	0
MN 300 cellulose	0	0
carboxy-methyl cellulose	0	0
lignine	0	0
vanillin	0	0
syringaldehyde	0	0

tetracycline, chlortetracycline, oxytetracycline), to the members of the streptomycin and neomycin groups and to polymyxin-B. It is interesting to note that REYES and TIEDJE (1976) studying the gut flora of the isopod *Tracheoniscus rathkei*, which feeds on decaying plant material, have obtained very similar data on the antibiotic sensibility of the intestinal bacterial population.

Table 2. The susceptibility to antibiotics of 90 strains representing the dominating bacterial fraction of the gut-community of *Eisenia lucens*

Antibiotics	Susceptible strains	
	No. of strains	Per cent of susceptible strains
Penicillin	1	1.2
Oxacillin	1	1.2
Methycillin	1	1.2
Chloramphenicol	57	63.3
Oleandomycin	0	0
Streptomycin	89	98.8
Tetracycline	88	97.7
Neomycin	89	98.8
Polymyxin-B	90	100
Erythromycin	21	23.3
Superseptyl	1	1.2
Nitrofurantoin	43	47.8
Chlortetracycline	90	100
Oxytetracycline	90	100
Vancomycin	1	1.2
Kanamycin	84	93.3
Spiramycin	7	7.8
Novobiocin	5	5.5
Ampicillin	1	1.2
Colistin	0	0
Lincomycin	0	0
Cephalosporin	3	3.4
Pristinamycin	0	0
Nalidixic-acid	72	80
Paramomycin	88	97.8
Gentamicin	89	98.8
Carbenicillin	50	55.6
Nystatin	0	0
0-129	1	1.2

All strains proved to be Gram-negative, forming short, straight or slightly curved rods about 0.4–0.6 by 0.4–2.0 μ m. The cells occur singly or in pairs, exhibiting considerable pleomorphism. They are motile usually by means of a single polar flagellum.

On synthetic agar plates the colonies of the majority of strains were smooth, moist, slightly yellowish, circular and opaque, with entire edges and with a dark center, 1–3 mm in diameter. Translucent, bluish, slightly raised colonies with entire edges were also observed. Some of the colonies were lobate-edged with a raspberry-like center. Water soluble exopigment was never observed in the media. The growth in liquid media is characterized in general, by moderate turbidity. Some strains were forming a pellicle in these media. The motility of the cells in wet-mount preparations examined by phase-contrast microscopy was typically zig-zag like. All the 92 strains studied utilized citrates and were catalase, oxidase and H_2S positive. Nitrites were produced from nitrates, but failed to produce indole. MR and VP tests were negative. The majority were growing at pH 9.5, and showed a positive urease reaction. Growth occurred in media with 0–4% NaCl. Starch and casein were not utilized. Proteolytic activity not present. No growth was found at 45 °C. Tweens were decomposed. All produced acid but no gas from dextrose, arabinose, xylose both aerobically and anaerogenically. They failed to attack sucrose. The fermentation of lactose, mannitol and the hydrolysis of aesculin varied from strain to strain.

The classification of these strains characterized by slightly curved Gram-negative, asporogenous, polarly flagellated rods brings out all the problems encountered in the taxonomy of the close genera *Pseudomonas*, *Aeromonas* and *Vibrio*, respectively, albeit there is no doubt that we can place them, already as a first approach, on the basis of the particular constellation of their diagnostic features, among the members of the genus *Vibrio*.

Their separation from the typical forms of pseudomonads seems to be clear, if we are considering their glucose-fermenting ability a distinguishing "key-character" of diagnostic value at the generic level (SHEWAN and VÉRON 1974). They are however hardly separated from the members of the genus *Aeromonas*. E.g. they were not sensitive (at least not on the employed medium) to pteridine (0—129; 20 µg pro disc) like the members of *Aeromonas*. In fact, some workers interested in the taxonomic differentiation of these genera found no evidence that the susceptibility of vibrios against 0—129 would be a distinguishing feature of general validity in the separation at generic level (COLWELL 1970; DAVIS and PARK 1962). On the other hand, LEE and his coworkers (1978) proved that in spite of the results of SCHUBERTS' testings (1962), only a sensitivity-test carried out with discs containing 150 µg pteridine could be a useful aid in separating the vibrios from aeromonads.

In other respects the growth of our strains in nutrient broth at pH 9.5 does confirm the opinion that they are true vibrios. Some of the investigators (SAKAZAKI 1963; DAVIS and PARK 1961) revealed that in contrast to vibrios most of the aeromonads are unable to grow at 9 or higher pH values.

The members of this vibrio population showed partly high salt tolerance. At many strains the upper limit for growth was at about 10 % NaCl, which is still considerably higher than that NaCl-concentration (about 4 %) which represents the maximum tolerable level for strains of *Aeromonas* spp.

Although having little taxonomic value, on synthetic agar medium the macroscopic morphology and appearance of colonies of the strains investigated gave a resemblance to the colonial characters of those enteropathogenic, facultatively halophylic bacteria which were identified by SAKAZAKI et al. (1963) as *Vibrio parahaemolyticus*.

Since nearly 75 % of the platable bacterial population of the hind-gut content of *E. lucens* is composed of the cells of this *Vibrio* sp. we can consider this organism as a predominant microbe in the *E. lucens*' intestinal bacterial community and as a true autochthonous species of the gut microbiota. Regarding its wide ranging biochemical capabilities (Table 1) we can suppose that it plays an important role in the degradation and transformation of food-materials. Further investigations is needed to clarify its particular location in the many possible gut microhabitats, its biochemical activities and precise systematic position.

4. Summary

Eisenia lucens, is an earthworm species of particular ecological behaviour. It tunnels in decomposing stumps, consuming wood materials rich in cellulose and lignins. This particular feeding habit is associated with a particular species composition of the microbial community in its gut. Seventy-three per cent of 473 bacterial strains isolated from samples of its gut content belong to the genus *Vibrio*. Ninety representative strains of this *Vibrio* sp. were selected for detailed comparative investigation. These strains are Gram-negative, facultatively anaerobic organisms forming rods (axis curved or straight) which are motile with a single polar flagellum. Citrates can be used as sole source of carbon. Glucose, arabinose and xylose are fermented. Only 21 strains of the 92 investigated are capable of producing acid from sucrose under anaerobic conditions. They are catalase, oxidase, urease and H₂S positive and methyl red, Voges-Proskauer and indole negative. They grow well at pH 9.5 and on mineral ammonium salt media with a simple carbon source. They are sensitive to antibiotics with a broad antibacterial spectrum. Some strains are also capable of growing still in about 10% NaCl concentration. Further investigations are necessary to determine their systematic position at species level.

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Address of the author: K. MÁRIALIGETI, Department of Microbiology, Eötvös L. University, Múzeum krt. 4/a., H - 1088 Budapest, Hungary.